

## RESEARCH PAPER

Journal of  
Biogeography

WILEY

# Himalayan wolf distribution and admixture based on multiple genetic markers

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## Funding information

Oxford-Lady Margaret Hall-NaturalMotion Graduate Scholarship

Handling Editor: Judith Masters

## Abstract

**Aim:** We explore the phylogeography of Himalayan wolves using multiple genetic markers applied on a landscape-scale dataset and relate our findings to the biogeographic history of the region.

**Location:** Himalayas of Nepal, the Tibetan Plateau of China and mountain ranges of Central Asia.

**Taxon:** Himalayan wolf (also called the Tibetan wolf), *Canis lupus chanco*.

**Methods:** We present a large-scale, non-invasive study of Himalayan wolves from across their estimated range. We analysed 280 wolf scat samples from western China, Kyrgyzstan and Tajikistan at two mtDNA loci, 17 microsatellite loci, four non-synonymous SNPs in three nuclear genes related to the hypoxia pathway, and ZF genes on both sex chromosomes.

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**Results:** Our results corroborate previous studies showing that the Himalayan wolf forms a distinct lineage adapted to high altitudes in excess of 4,000 m elevation. We found a correlation between hypoxia adaptation and the divergent Himalayan wolf mtDNA haplotype found across the Tibetan Plateau of Qinghai, the Tibetan Autonomous Region and the Nepalese Himalayas. We identified a region of admixture between Himalayan and grey wolves at the boundary of their distributions, where the Tibetan Plateau elevation gradually drops.

**Main Conclusion:** Based on multiple genetic markers, the Himalayan wolf forms a reciprocally monophyletic lineage with a unique adaptation to high altitude. We propose that the divergence of the lineage is related to past uplift of the region, and that the lineage maintains its high-altitude niche, in part, by means of its genetic adaptation to hypoxia. We conclude that the Himalayan wolf merits taxonomic recognition and designation as an evolutionary significant unit (ESU).

#### KEYWORDS

admixture, *Canis lupus chanco*, China, Himalayan wolf, Nepal, phylogeography, population structure, Tibetan Plateau

## 1 | INTRODUCTION

The Himalayan wolf (also called the Tibetan wolf) has been shown to comprise a genetically distinctive lineage from the Holarctic grey wolves (Aggarwal, Kivisild, Ramadevi, & Singh, 2007; Sharma, Maldonado, Jhala, & Fleischer, 2004; Werhahn et al., 2018; Werhahn, Senn, et al., 2017). In mitochondrial DNA (mtDNA) analysis, Himalayan wolves constitute a monophyletic clade that is sister to the Holarctic grey wolf subspecies (Aggarwal et al., 2007; Koepfli et al., 2015; Sharma et al., 2004; Werhahn et al., 2018), whereas nuclear genome analysis (Fan et al., 2016) indicates that they form a clade within the Holarctic grey wolf complex. Himalayan wolves exhibit zinc finger (ZF) protein gene haplotypes on both sex chromosomes that differ from those found in Holarctic grey wolves. Specifically, the ZF protein gene haplotype on the Himalayan X chromosome is shared with the African wolf, recently posited as *Canis lupaster* (Álvares et al., 2019; Viranta, Atickem, Werdelin, & Stenseth, 2017), while the ZF haplotype on the Himalayan Y chromosome is unique among wolf lineages (Werhahn et al., 2018; Werhahn, Senn, et al., 2017). The Himalayan lineage exhibits mutations on four non-synonymous SNPs in three functional nuclear genes related to the hypoxia pathway (vonHoldt, Fan, Vecchy, & Wayne, 2017; Werhahn et al., 2018; Zhang et al., 2014), which are likely to affect high-altitude adaptation, that is, the evolution of physiological mechanisms to cope with lifelong hypoxic conditions in high-altitude habitats (Storz, Scott, & Cheviron, 2010). Similar hypoxia adaptations have been found in the Tibetan people that share these high-altitude ecosystems (Beall, 2007; Huerta-Sanchez, 2014; Peng et al., 2011; Yi et al., 2010), as well as in domestic yaks (*Bos grunniens*) (Qiu et al., 2012) and Tibetan mastiff dogs (*Canis lupus familiaris*). The latter species may have acquired them via hybridization with Himalayan wolves (Li et al., 2014).

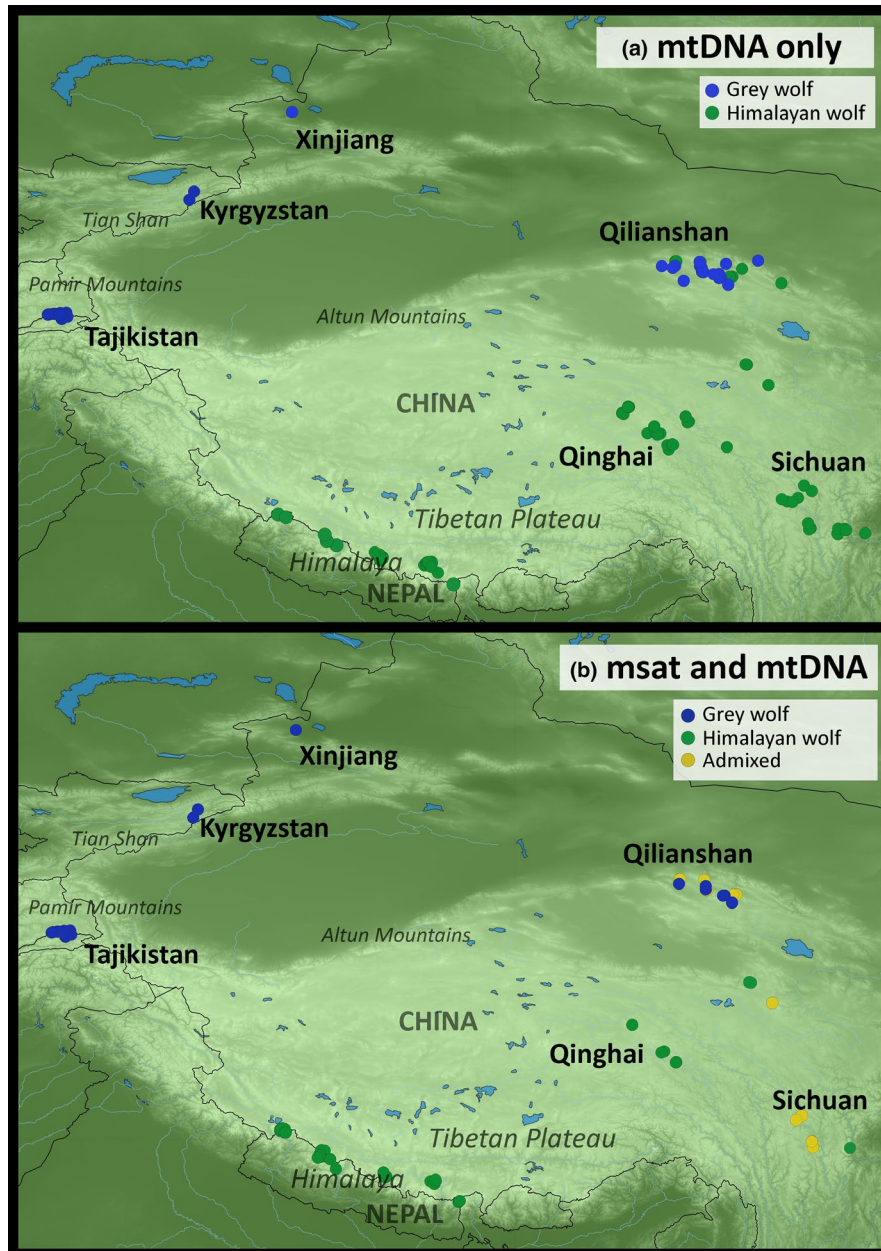
Estimates of divergence dates between Himalayan and other wolves vary depending on the sequences used in the calculation. Mitochondrial sequences place this split between 0.55–0.8 Ma (Matsumura, Inoshima, & Ishiguro, 2014; Sharma et al., 2004; Werhahn et al., 2018), prior to the radiation of modern grey wolves estimated at 0.5–0.3 Ma (Sotnikova & Rook, 2010; Tedford, Wang, & Taylor, 2009). In contrast, Fan et al. (2016), using nuclear genomic data, estimated much more recent divergence times for wolves, projecting the split of Eurasian and North American wolves at 12,500 years before present, followed by the divergence of Eastern (including Himalayan) and western Eurasian wolves at 11,700 years ago.

Himalayan wolves were first characterized genetically using samples deriving from the Himalayas (Aggarwal et al., 2007; Sharma et al., 2004), but later evidence demonstrated that their range included the alpine grassland and tundra habitats of the Tibetan Plateau (Werhahn et al., 2018; Werhahn, Senn, et al., 2017). Authors have deployed various scientific names for this lineage in different studies and databases, including *C. l. chanco*, *C. l. laniger*, *C. l. himalayensis* and *C. l. filchneri*, with both Himalayan wolf and Tibetan wolf as common names (Aggarwal et al., 2007; Bocci, Lovari, Khan, & Mori, 2017; Chetri et al., 2016; Gray, 1863; Hodgson, 1847; Matschie, 1908; Sharma et al., 2004; Shrotryia, Lyngdoh, & Habib, 2012; Werhahn, Kusi, Sillero-Zubiri, & Macdonald, 2017; Werhahn et al., 2018; Werhahn, Senn, et al., 2017). Recently, Álvares et al. (2019) recommended the acceptance of *Canis lupus chanco* and Himalayan wolf, and we follow this terminology here. We emphasize, however, that this population is distinguished by a specialized high-altitude feeding ecology (Werhahn et al., 2019), and a vocalization that can be differentiated from those of Holarctic grey wolves (Hennelly, Habib, Root-Gutteridge, Palacios, & Passilongo, 2017).



Members of the genus *Canis* hybridize readily under favourable circumstances and disperse over large distances, two characteristics that facilitate gene flow and are responsible for the relatively low levels of population diversification in Holarctic grey wolves (Dufresnes et al., 2019; Fan et al., 2016; Pilot et al., 2010; Vilà et al., 1999). Gopalakrishnan et al., (2018) highlighted the role of interspecific hybridization in the evolution of the canid family, especially within the genus *Canis*. The distinctiveness of the Himalayan wolf despite of its

geographic proximity to the widely distributed Holarctic grey wolf (Boitani, Phillips, & Jhala, 2018) is therefore of particular interest. In this contribution, we enhance the general understanding of canid evolution by providing landscape genetic data pertinent to the distribution of Himalayan wolves and their admixture with grey wolves. We explore possible mechanisms behind the separation of the Himalayan lineage to aid accurate systematic assessments and conservation planning.



**FIGURE 1** Map showing the region of the Himalayas and the Tibetan Plateau with study areas and specific sample locations. (a) Shows the mtDNA of samples, and (b) shows the microsatellite and mtDNA results combined per sample. The two maps illustrate that admixed individuals are found in the north-eastern edge regions of the Tibetan Plateau, that is, in Qilianshan mountains and Sichuan, where the habitat gradually changes to lower lying habitats where grey wolves predominate. (c) Hypoxia pathway-related SNPs per individual sample and geographic origin. Letters in circles indicate SNP genotypes of three hypoxia-related genes, and circle sizes indicate the number of samples bearing the genotypes. Clockwise from top right of each circle: *ANGPT1*, *EPAS1*, *RYR2-1* and *RYR2-2*. Dark green represents homozygous alleles characteristic of Himalayan wolves, and pale green signifies heterozygous alleles present in this population. Grey wolf alleles are shown in blue, and white indicates missing data (for full details see Table S5)



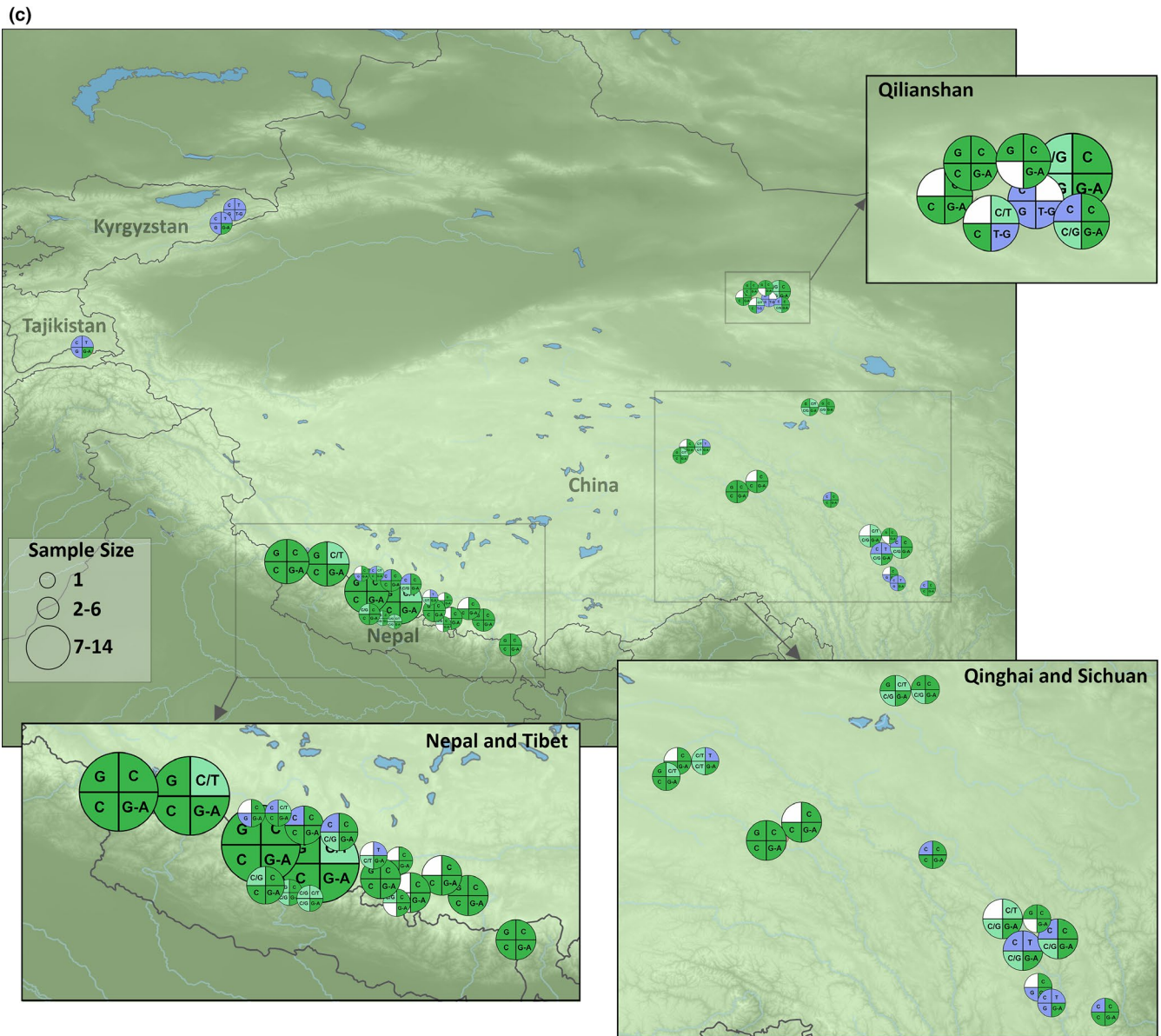


FIGURE 1 (Continued)

## 2 | MATERIALS AND METHODS

### 2.1 | Study areas

We collected samples for this study across multiple areas of the Asian high mountain landscape, including the Tibetan Plateau. Collection sites included Sanjiangyuan National Nature Reserve (Zhaqing and Namsai Township, Zadoi County, Yushu Prefecture, Qinghai Province); Qomolangma National Nature Reserve in the Tibetan Autonomous Region (TAR); Qilianshan mountains (Gansu and Qinghai Provinces) and Sichuan. We further sampled different mountainous areas of Central Asia, including Santai National Forest in Bortala Mongol Autonomous Prefecture (Xinjiang Autonomous Region, China); Zorkul Reserve of Tajikistan and Jeti-Oguz region of Kyrgyzstan (Figure 1; Table 1). To enhance our database, we included previously published data from three

study areas in the Himalayas of Nepal (see Werhahn et al., 2018). Tibetan Plateau habitats consisted of alpine tundra and grassland, while those in Tajikistan and Kyrgyzstan comprised alpine steppe and wetlands. The area of Bortala, Xinjiang, was made up of open grassland, sparse shrubs and coniferous forest. The primary carnivore species in the region, in addition to grey and Himalayan wolves, included snow leopard (*Panthera uncia*), red fox (*Vulpes vulpes*), Tibetan fox (*V. ferrilata*), Pallas's cat (*Otocolobus manul*), Eurasian lynx (*Lynx lynx*), brown bears (*Ursus arctos*) and domestic dogs (*Canis lupus familiaris*).

### 2.2 | Collection of genetic material

We collected putative wolf scat samples along opportunistic transects, focusing on ridges, streams and valley floors in all study areas,

as these topographic features are important traveling routes for carnivores. We noted GPS position and associated habitat features for each sample. Detailed information on samples is presented in Table S4.

## 2.3 | Genetic analysis

Two partial mtDNA loci were examined, D-loop (242 bp) and cytochrome *b* (508 bp), as they have previously been shown to be appropriate for separating wolf lineages (Werhahn et al., 2018). In total, 280 wolf samples were successfully sequenced at these mtDNA loci following the methods and PCR conditions reported in Werhahn, Senn, et al. (2017). A subset of 110 samples were successfully genotyped at 17 microsatellite loci previously screened as Himalayan wolf, using the same methods as reported in Werhahn et al. (2018). Of these, 18 samples had to be discarded owing to missing data, and 6 were later discarded as duplicate genotypes (i.e. scats from the same individual), resulting in 86 microsatellite genotypes from across the Tibetan Plateau of China, Tajikistan and Kyrgyzstan. This dataset was then combined with the 37 samples of Himalayan wolf from Nepal, European and Mongolian grey wolf, and domestic dogs published in Werhahn et al. (2018) to provide a comprehensive microsatellite dataset for the Himalayan wolf across the sampled range.

For the zinc finger (ZF) analysis we developed new, shorter primers to improve success rate (Table S9), adopting the laboratory procedures described in Werhahn, Senn, et al. (2017). For the hypoxia pathway-related SNPs we looked specifically at the *ANGPT1*, *EPAS1*, *RYR2-1* and *RYR2-2* genes (Werhahn et al., 2018; Zhang et al., 2014), using the methods reported in Werhahn et al. (2018). We used Geneious version 8.1.8 for editing, quality control, building Bayesian phylogenies from mtDNA sequences and scoring the 17 microsatellites. For the Bayesian phylogeny reconstruction, we included wolf reference samples of

partial and full mitochondrial genomes obtained from GenBank (see Tables S2 and S3). We conducted a genetic distance analysis comparison including coyotes (*Canis latrans*, GenBank Accessions: DQ480510 and KF661096), grey wolves from Europe and North America (GenBank Accessions: KF661089, KF661091, KU696409, KF661095, KU696411, KF661087, KF661076, KF661056 and KF661090), grey wolves from Mongolia, Inner Mongolia and Xinjiang (China) (GenBank Accessions: SRR2017905, KU696393, KU696392, KU696396, GQ374438, SRR20179, KU696395 and KU693394), Himalayan wolves (GenBank Accessions: FJ032363, NC011218, EU442884 and KF573616), African wolves (GenBank Accessions: KT378605 and NC027956), Iberian wolves (*C. l. signatus*, GenBank Accessions: KU644670) and Indian wolves (*C. l. pallipes*, GenBank Accessions: KF661043 and KF644666) based on full mtDNA using the software MEGA (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

To analyse the microsatellite data we used the Genalex 6.503 plug-in for Microsoft Excel for basic quality control, diversity statistics, Hardy-Weinberg testing and calculating genetic distances between locations (Peakall & Smouse, 2006, 2012). The R packages *allelematch* and *adegenet* (Galpern, Manseau, Hetting, Smith, & Wilson, 2012; Jombart, 2008; Jombart & Ahmed, 2011; R Core Team, 2013) were used for clustering the microsatellite genotypes. The software package *STRUCTURE* (Pritchard, Stephens, & Donnelly, 2000) was used to estimate admixture among the different wolf populations. We used a burn-in of 500,000 and MCMC replicates of 1,000,000 after burn-in with three replicates for each tested K to check for stability. The optimal population number K was estimated by plotting K versus Ln P(D) and the Evanno method run with 15 replicates for K = 2–9 (Evanno, Regnaut, & Goudet, 2005). The software *HP-Rare* (Kalinowski, 2005) was used to calculate allelic richness for the 17 microsatellite loci. We conducted a Fisher's Exact Test for Count data to check correlation between mtDNA lineage and the four hypoxia pathway-related SNPs.

**TABLE 1** Study areas with sample size, year collected, habitat type, average elevation and literature if previously published

Study region	Samples	Year collected	Habitat type	Elevation (m)	Literature
Sanjiangyuan (Qinghai, China)	165	2018, 2014	Alpine grassland and steppe	4,404	
Tibet (TAR, China)	93	2015–2017	Alpine grassland and steppe	4,650	
Qilianshan Mountains (China)	43	2014–2017	Grassland, shrub land and coniferous mixed forest	3,725	
Sichuan (China)	73	2014–2017	Forest (oak-conifer) and grasslands	3,929	
Bortala (China)	7	2016	Grassland, shrubs and coniferous forest	525	
Jeti-Oguz (Kyrgyzstan)	6 (incl. 4 from (Werhahn et al., 2018))	2017	Alpine grassland and steppe	3,107	Werhahn et al. (2018)
Zorkul (Tajikistan)	30	2017	Alpine grassland and steppe	4,294	
Himalayas (Nepal)	53	2015–2016	Alpine grassland and steppe	4,813	Werhahn et al. (2018)

Note: Of the total of 470 collected samples, 280 samples were from wolves and were successfully sequenced.

### 3 | RESULTS

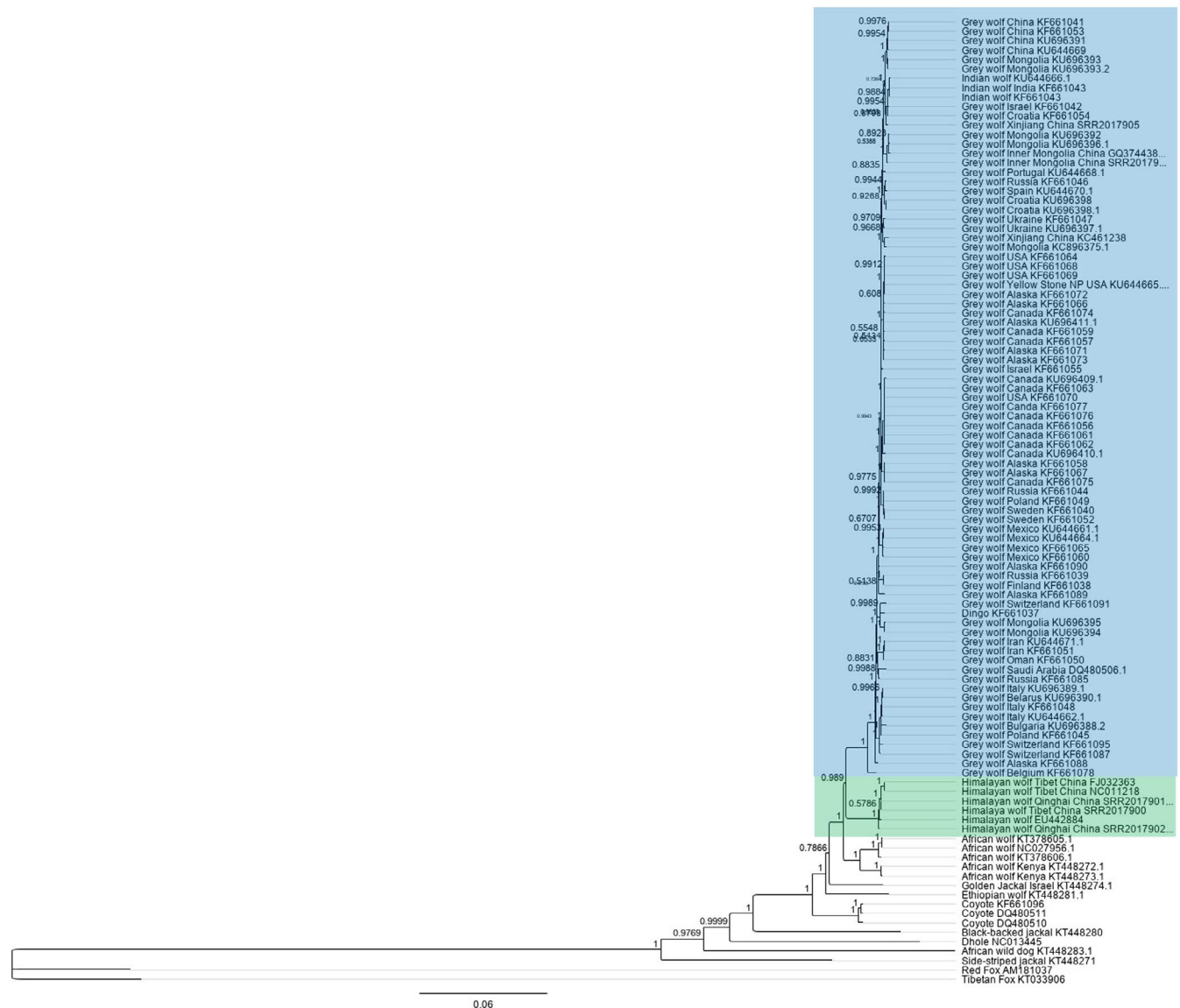
### 3.1 | Mitochondrial DNA and nuclear microsatellites

In agreement with the findings of Werhahn et al. (2018) concerning Himalayan wolves from Nepal, Himalayan wolves from TAR and Qinghai showed divergent mtDNA haplotypes with respect to the 242 bp D-loop fragment, the 508 bp cytochrome *b* sequence and the full mitochondrial genome. The lineage was supported in Bayesian reconstructions based on D-loop (posterior probability 1), cytochrome *b* (posterior probability 0.8443, Figure S1) and full mtDNA (posterior probability 0.989, Figure 2) sequences. Himalayan wolf mtDNA haplotypes were prevalent across the Himalayas and the Tibetan Plateau (Figure 1a). When combining the mtDNA and microsatellite data, we found a consistent pattern of admixed individuals (Figure 1b) at the edges of the range. All admixed animals had

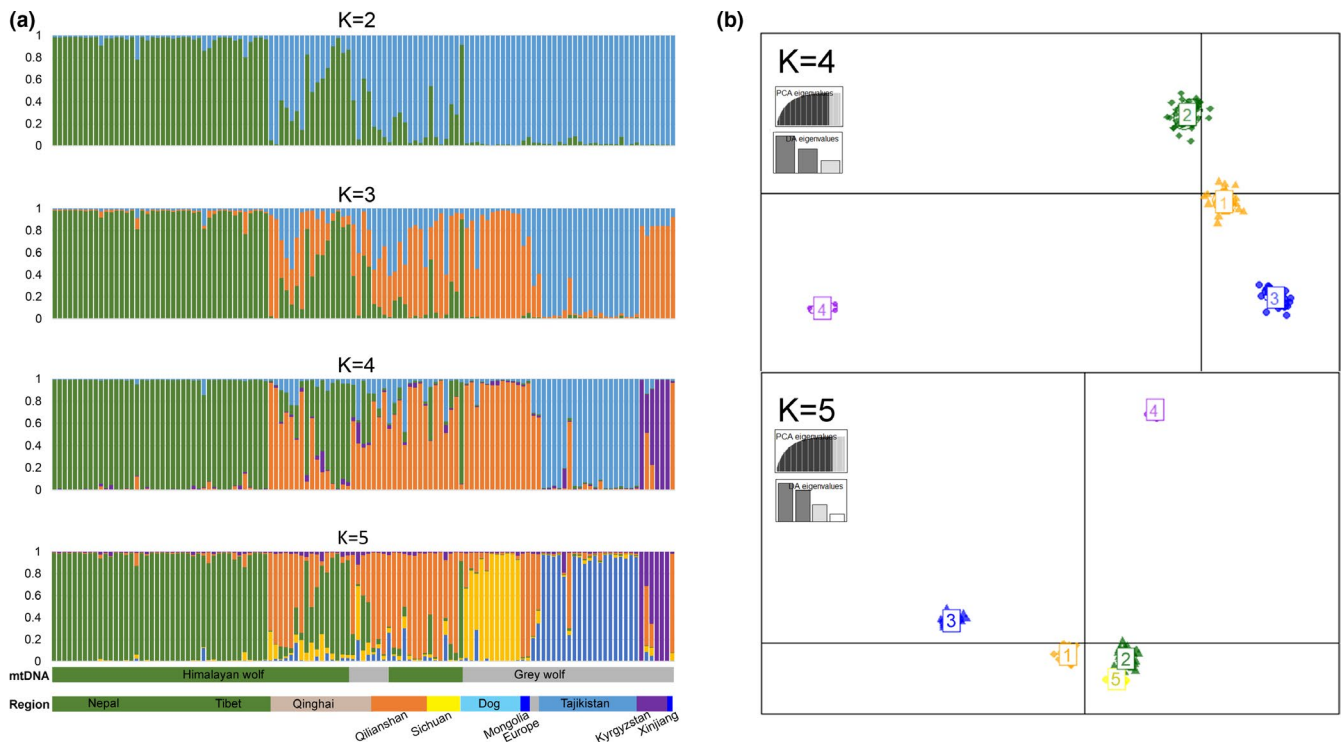
Himalayan wolf mtDNA haplotypes, whereas their microsatellite allelic patterns revealed admixture between Holarctic and Himalayan wolves (Figure 3). Structure analysis of microsatellite data from individuals sampled across the Himalayas, the Tibetan Plateau and the surrounding mountain ranges in Tajikistan and Kyrgyzstan suggested an optimal  $K = 5$  (Figure 3). Genetic distances (Nei unbiased) between locations are shown in Table 2 and allelic richness in Table S7. Table S8 presents full details regarding allele frequency per population.

### 3.2 | Hypoxia adaptation

The Himalayan wolf lineage predominated above 4,000 m elevation, whereas grey wolves were present at lower elevations (Figure 4). We successfully amplified four SNP loci of hypoxia pathway-related



**FIGURE 2** Canid phylogeny based on full mitochondrial genomes with GenBank accession numbers (also see Figure 4 in Werhahn et al. (2018))



**FIGURE 3** (a) Structure plot of microsatellite results for  $K = 2-5$ , with  $K = 5$  receiving most support. At the bottom, the mtDNA lineage and geographic origin of samples are provided. (b) The corresponding Discriminant Analysis of Principal Components (DAPC) plot with the following geographic origin of wolves at  $K = 4$ : (1) Qinghai, Sichuan, Qilianshan, Xinjiang, Mongolia and Europe and including the Tibetan mastiff dogs; (2) Nepal and Tibet; (3) Tajikistan; (4) Kyrgyzstan. At  $K = 5$  the clusters represent wolves from: (1) Qinghai, Sichuan, Qilianshan; (2) Nepal and Tibet; (3) Tajikistan; (4) Kyrgyzstan; (5) Europe, Mongolia, Xinjiang, Qinghai, Sichuan and including Tibetan mastiff dogs

**TABLE 2** Nei unbiased genetic distances among wolf populations derived from 17 microsatellites and grouped by geographic region

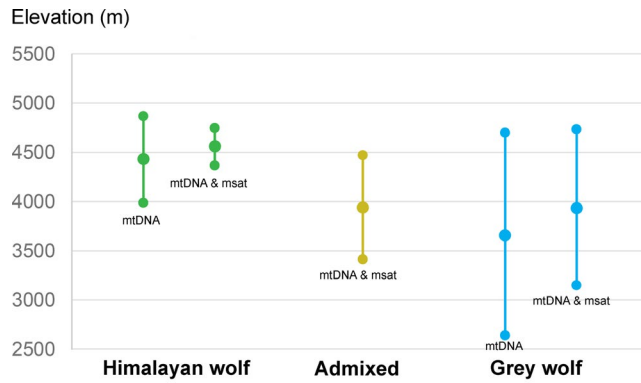
Nepal & Tibet ( $N = 43$ )	Dogs (Tib. Mastiff, $N = 11$ )	Mongolia & Xinjiang ( $N = 3$ )	Europe ( $N = 2$ )	Kyrgyzstan ( $N = 6$ )	Qilianshan ( $N = 11$ )	Qinghai ( $N = 21$ )	Sichuan ( $N = 7$ )	
0.105	0.000							Dog (Tib. Mastiff)
0.215	0.307	0.000						Mongolia & Xinjiang
0.365	0.424	0.193	0.000					Europe
0.252	0.250	0.516	0.430	0.000				Kyrgyzstan
0.296	0.231	0.634	0.806	0.448	0.000			Qilianshan
0.135	0.199	0.256	0.333	0.277	0.213	0.000		Qinghai
0.498	0.567	0.738	0.537	0.431	0.756	0.349	0.000	Sichuan
0.415	0.588	0.664	0.731	0.506	0.638	0.261	0.233	Tajikistan

Note: Sample sizes ( $N$ ) are in parentheses.

genes for 59 fecal samples from TAR, Sichuan and Qinghai in China, and Tajikistan and Kyrgyzstan (Figure 1c; Table S4). When considering the hypoxia pathway results combined with the mtDNA and microsatellite data of the same individuals, we found that the hypoxia adaptation present in the Himalayan wolves of Nepal and TAR appeared to be linked to a diagnostic mtDNA Himalayan wolf haplotype. Fisher's Exact Test for Count data showed significant correlation between the alleles at each of the four hypoxia-related SNPs and the divergent Himalayan wolf mtDNA haplotypes (ANGPT1:  $p$ -value =  $2.072e^{-1}$ ; EPAS:  $p$ -value = .0018; RYR2-1:

$p$ -value <  $2.2e^{-16}$ ; RYR2-2:  $p$ -value =  $3.264e^{-07}$ ). Our results further showed that admixed individuals, characterized by Himalayan wolf mtDNA and intermediate nuclear DNA, shared the hypoxia adaptation, especially on the EPAS gene (Figure 1). Some admixed individuals showed grey wolf variants at particular loci, but usually only one gene had a characteristically grey wolf allele while the rest presented the Himalayan wolf hypoxia adaptation (Figure 1c; Table S5). We also plotted each hypoxia gene allele against the elevation of the individuals sampled (Figure 5) to gain additional insight into potential selection at each locus.





**FIGURE 4** Mean altitude with standard deviation of genetically verified Himalayan wolves, admixed individuals and grey wolves sampled in China, Tajikistan and Kyrgyzstan. Admixed individuals are characterized by Himalayan wolf mtDNA and intermediate microsatellites

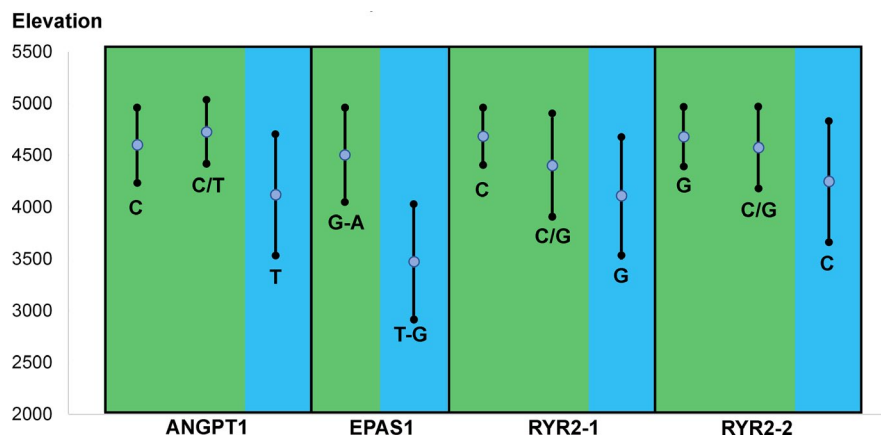
### 3.3 | Zinc finger results

We successfully amplified 14 fecal samples for ZFY and 49 for ZFX from TAR, Qinghai and Sichuan (China) and Tajikistan. The results from wolves in TAR and Qinghai corroborated the previously described, unique Himalayan wolf haplotypes for both the ZFX and ZFY (Werhahn et al., 2018), with a few exceptions that may indicate introgression (see Table S6). Exceptions to the ZFY pattern included one Himalayan wolf from Qinghai with a grey wolf haplotype; one admixed individual from Qinghai with a Himalayan wolf haplotype and another admixed individual from Qinghai with a grey wolf haplotype. The ZFX exceptions included one Himalayan wolf from Qinghai and two admixed individuals from Qinghai and Sichuan with grey wolf haplotypes; and two admixed individuals from Qinghai and Sichuan, two admixed individuals from Qilianshan and two grey wolves from Qilianshan and Qinghai with Himalayan wolf haplotypes.

## 4 | DISCUSSION

In general agreement with other wolf phylogeographic studies (Ersmark et al., 2016; Fan et al., 2016; Meng, Zhang & Meng, Zhang, & Meng, 2009; Pilot et al., 2010; Rueness et al., 2011; Zhang et al., 2014), our landscape-scale analysis demonstrated consistent genetic differentiation across multiple markers between Himalayan wolves from TAR, Qinghai and the Himalayas and adjacent grey wolf populations. However, the level of independence of the Himalayan lineage has been difficult to assess for several reasons. First, many recent, large-scale phylogeographic studies of Holarctic wolves have not included samples from high-altitude Asian sites (e.g. Gopalakrishnan et al., 2018; Loog et al., 2018; Pilot et al., 2019). In studies where high-altitude samples have been included, reconstructions based on mitochondrial sequences (Aggarwal et al., 2007; Koepfli et al., 2015; Sharma et al., 2004; Werhahn et al., 2018; Werhahn, Senn, et al., 2017) have generated different patterns of relationship from those based on nuclear genomes (Fan et al., 2016). Mitochondrial and nuclear genomes follow different paths of inheritance (maternal vs. biparental, respectively; Gopalakrishnan et al., 2018; de Groot et al., 2016), and have different mutation rates, and thus track different genetic processes contributing to evolutionary divergence (e.g. see Cahill et al., 2013). Whereas our mitochondrial study supported an early divergence for the Himalayan wolves, making them the sister taxon to the grey wolves, the nuclear genome study of Fan et al. (2016) indicated a recent ancestry for all extant wolf lineages, and reconstructed Himalayan wolves as a recent subclade within the Holarctic grey wolf complex. Thalmann et al., (2013), in their global analysis of wolves and dogs, excluded two Himalayan wolf samples from their analyses 'since their phylogenetic positioning suggests only a distant relationship to all extant grey wolves'.

In addition to our phylogeographic study, the nuclear genomic markers we analysed (SNPs on the functional genes involved in hypoxia adaptation, the ZF protein gene on the Y and X sex chromosomes) were different among Himalayan wolves, and the



**FIGURE 5** Mean and standard variance of elevation for wolf samples carrying different alleles of hypoxia pathway-related genes. For EPAS1 the allele combination for the two regions tested were fixed, that is, individuals with the hypoxia adaptation consistently showing alleles G and A, whereas grey wolves lacking the adaptation carried alleles T and G. Heterozygotes are indicated by a slash (e.g. C/T) signifying the presence of both alleles. More information is shown in Table S5





microsatellite data, which provide insight into population structure, also distinguished the Himalayan lineage. It is possible that the individuals sampled in the study by Fan et al. (2016) were of admixed origin, explaining their position within the grey wolf clade. This interpretation receives support from our detection of a hybrid zone at the edges of the Himalayan wolf range, and from our observation that admixed individuals have both mtDNA and hypoxia pathway-related SNPs characteristic of Himalayan wolves, but nuclear DNA profiles intermediate with grey wolves. The genomic data for the 'Highland wolves' included in the Fan et al. (2016) study are based on two samples from Tibet and two from Qinghai (the same samples as were used by Zhang et al., 2014). The samples came from captive wolves (Luobulingka Zoo in Tibet and Xining Zoo in Qinghai), but were wild-born (Supporting Information, Zhang et al., 2014). In the light of our results, the provenances of these animals are highly relevant for understanding their phyletic placement. In both cases, the likely source areas of the zoo animals lie within zones of admixture (Qinghai, including Xining, situated at 2,270 m elevation; Luobulingka Zoo in Lhasa, Tibet, situated at 3,650 m elevation) (Figure 1).

Our mapping of zones of admixture and our insights into the genetic profiles of admixed individuals should be considered in future genomic studies. Specifically, future work should include full-genome data from multiple contemporary wolf individuals of verified geographic origin, both from within the Himalayan wolf core distribution and from the admixture zone at the distribution edges. Use of single/ limited numbers of samples to infer population measures should be avoided due to the potential confounding impact of admixture which is clearly present.

Our data indicate that the Himalayan wolf lineage occurs across the continuous high-altitude habitats of the Himalayas and the Tibetan Plateau, whereas the Holarctic grey wolf occupies the surrounding lower elevation regions of Kyrgyzstan, Mongolia and Eastern China. Our results, combined with those of previous studies (Sharma et al., 2004; Werhahn et al., 2018), indicate that the core distribution of the Himalayan wolf is in the high Himalayas of Nepal and India (its presence in Bhutan remains to be verified), and spreads across the Tibetan Plateau and large parts of Qinghai, with a prevalence in habitats above 4,000 m.

#### 4.1 | High-altitude adaptation

The low oxygen availability at high altitudes presents environmental stress in the form of severe, lifelong high-altitude hypoxia (Dosek, Ohno, Acs, Taylor, & Radak, 2007). This constitutes a constant selective pressure that has shaped the genomes of species inhabiting these ecosystems and led to functional and physiological adaptations to cope with the conditions (Polle & Rennenberg, 1992). In mammals, including humans, exposure to high altitudes decreases partial pressure of oxygen and increases formation of reactive oxygen and nitrogen species (RONS), which cause oxidative damage to lipids, proteins and DNA (Maiti et al., 2006). Adaptive responses have fine-tuned the physiological mechanisms that mitigate the destructive effects of free radicals (Maiti et al., 2006). However, the genetic mechanisms underpinning

long-term survival of humans in these extreme environments remain poorly understood (Moore, 2001; Peng et al., 2011; Simonson et al., 2010). The situation is similar for the Himalayan wolf, as the genetic and physiological mechanisms involved in high-altitude adaption have only recently been revealed (vonHoldt et al., 2017; Werhahn et al., 2018; Zhang et al., 2014). These wolves range mostly above 4,000 m, an elevation at which less than 12.7% effective oxygen is available, in contrast to the 21.9% available at sea level (West, Schoene, & Milledge, 2007). In line with previous work (vonHoldt et al., 2017; Werhahn et al., 2018; Zhang et al., 2014), our study of Himalayan wolves from across the Tibetan Plateau showed differentiated alleles on four SNPs of three functional hypoxia pathway-related genes: EPAS1 and ANGPT1, which increase oxygen delivery, and RYR, which strengthens heart function (Zhang et al., 2014). These functional mutations were correlated with the Himalayan wolf mtDNA haplotypes and indicate an adaptive advantage for Himalayan wolves in hypoxic environments. Tibetan mastiff dogs, the characteristic breed of these high-altitude environments, have probably acquired high-altitude adaptation through introgression from wolves to dogs (Li et al., 2014; vonHoldt et al., 2017). In our samples, wolves and dogs from the Himalayas and the Tibetan Plateau, as well as admixed individuals, all had the same hypoxia-adapted allele at the EPAS1 gene (Table S5). The admixed individuals at the distribution edges shared the mtDNA of Himalayan wolves and tended to have hypoxia adaptation through fixation of the EPAS1 gene, corroborating the findings of Gou et al. (2014) and vonHoldt et al. (2017). The grey wolves in Kyrgyzstan and Tajikistan showed little indication of admixture with Himalayan wolves, but showed introgression on hypoxia pathway-related genes, especially EPAS1. This introgression is likely to be adaptive, as these wolves live at higher elevations with some degree of connectivity with the Himalayas and the Tibetan Plateau. Microsatellite data revealed that Himalayan wolves in Nepal and TAR show very limited admixture, whereas populations towards the margins of the Tibetan Plateau (i.e. northern Qinghai, Qilianshan and Sichuan) at lower elevations showed signs of admixture with grey wolves. The distribution to the south is likely to be more marked because of the steep habitat cline from the Himalayas to the lowlands of the Indian subcontinent. Admixture analysis of nuclear microsatellite data at  $K = 2$  suggested there is little gene flow between Himalayan and grey wolves. At  $K = 3-5$  the wolves from Tajikistan and Kyrgyzstan and domestic dogs split successively from the grey wolf complex.

#### 4.2 | Speciation and biogeography

The area occupied by Himalayan wolves has a complex topography. This was a result of major orogenic events initiated during the Eocene epoch, when India and Asia collided, and the Himalayas and the Tibetan Plateau were uplifted. One of the last widespread, rapid uplift episodes of the Qinghai-Tibetan Plateau, the Kunlun-Huanghe Tectonic Movement, took place between 1.1 and 0.6 Ma, when glaciations developed around the high mountains on the Tibetan Plateau, but not over the entire plateau surface (Li & Fang, 1999; Li et al., 2014; Zhou, Wang, Wang, & Xu, 2006). Complex topographies interact with

global changes in climate to isolate populations and create new ecological conditions, facilitating speciation through divergent selection and adaptation (Liu et al., 2013). The Pleistocene epoch (2.588–0.0117 Ma) was characterized by cycles of glaciation, which dramatically reshaped global biodiversity patterns, eliminating terrestrial biota from many mid- to high-latitude areas of the planet (Wallis, Waters, Upton, & Craw, 2016). Mitochondrial estimates of the divergence of the Himalayan and grey wolves range from 0.8 to 0.55 Ma (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018), a period that coincides with both geological and climatic disruptions. These events may have isolated the ancestral Himalayan wolf population and led them to diverge from the ancestors of the wolf-dog clade (Matsumura et al., 2014; Sharma et al., 2004; Werhahn et al., 2018). Similarly, other taxa in the region, such as Himalayan brown bears (*Ursus arctos isabellinus*), show diversification at a similar time period (Lan et al., 2017).

### 4.3 | Speciation by environmental cline

Kawecki (1997) states that the evolution of lineages can be driven by genetic variation due to genetic loci that affect fitness in one habitat and are neutral or nearly so in others, such as the genes suspected to be responsible for hypoxia adaptation in the Himalayan wolf. Furthermore, speciation can be facilitated through strong habitat clines (Doebeli & Dieckmann, 2003), such as the dramatic altitudinal difference that separates the Tibetan Plateau and Himalayas from the surrounding, lower-lying regions. The related change in climate, habitat type, prey composition and geographic distance contribute to isolation, genetic drift and selective divergence as the lineages evolve independently (Geffen, Anderson, & Wayne, 2004; Leonard, 2014; Pilot, 2006).

We propose that the high-altitude environment was an important source of selection leading to the divergence of the Himalayan wolf lineage and its unique genetics which is interesting in the light of two prominent wolf characteristics: that is, long dispersal distances (over hundreds of km, with 1,000 km documented; Ciucci, Reggioni, Maiorano, & Boitani, 2009) and ready hybridization with other wolf-like canids when conspecifics are lacking (Kusak et al., 2018; Pacheco et al., 2017). Both of these behaviours can lead to polyphyletic lineages and homogenization of populations through gene flow. We hypothesize that the Himalayan wolf's specific genetic adaptation to life in the extreme high altitudes gives it an adaptive advantage and fitness benefit over grey wolves in these environments.

### 4.4 | Admixture at the distribution boundaries

Hybridization is ubiquitous in nature (Stanton et al., 2019). It occurs between wild animals and their domestic relatives and among related wild species (Adams, Kelly, & Waits, 2003; Pacheco et al., 2017; Randi, 2008), with the latter having an important role in the evolution of the canid family (Gopalakrishnan et al., 2018; Pilot et al., 2019). Himalayan wolves show a consistent genotype of hypoxia adaptation across Nepal, TAR and Sichuan, whereas grey wolves in Qilianshan do not

show this adaptation. Our results indicated that the Himalayan wolf mtDNA haplotype is linked to the genetic hypoxia adaptation. We found admixed individuals at the edges of the Tibetan Plateau, that is, in the Qilianshan Mountains (Gansu and northern Qinghai province) and Sichuan province, which showed mtDNA haplotypes and hypoxia adaptation of Himalayan wolves, but microsatellite profiles of Himalayan grey wolf intermediates. These admixed individuals may benefit from maintaining the mtDNA and hypoxia genes of Himalayan wolves, but there is no evidence whether the hypoxia adaptation is neutral or negative at lower elevations.

Historically, wolves were present in most of continental China (Wang et al., 2016), and the admixture in Sichuan may originate from grey wolves in the lower-altitude areas of eastern and south-eastern China. Northern Qilianshan is characterized by lower elevations with open grasslands and desert areas, where we found only grey wolves and admixed individuals (Figure 1). In these habitats, the hypoxia adaptation may be less advantageous, and introgression may originate from grey wolves in inner and northern Mongolia, or from dispersal through the Altun Mountains connecting Qilianshan and the mountains of Central Asia (i.e. the Tian Shan and Pamir Mountains in Tajikistan and Kyrgyzstan). The presence of both admixed individuals and grey wolves in Qilianshan (lower-elevation habitats), versus only admixed individuals in Sichuan (high-elevation habitats) points to the importance of elevation to the presence of Himalayan wolves.

The width of an admixture zone is assumed to be a function of the distance travelled between the places of birth and first reproduction, and the degree of selection against admixed individuals (Barton & Hewitt, 1985, 1989; Wayne, Geffen, & Vilà, 2004). Baker and Bradley (2006) proposed that two taxa represent different species when hybridization is restricted to a limited geographic area, while outside this area the taxa represent well-supported monophyletic clades in terms of mitochondrial and nuclear genetic variation. Our preliminary results suggest that the admixture zone between Himalayan and grey wolves is 660–900 km wide, but probably varies with topography and elevation. Admixed individuals facilitate gene flow and introgression in the contact zones between taxa (Jeong et al., 2014), and their significance for adaptation and speciation implies that they should be considered in the delineation of protected areas (IUCN, 2016).

**TABLE 3** Net percentage genetic distances among different canid lineages based on the full mitochondrial genome calculated with MEGA

	Holarctic grey wolf (N = 9)
Coyote (N = 2)	4.15
African wolf (N = 3)	2.55
Himalayan wolf (N = 4)	2.45
Grey wolf Mongolia (N = 8)	0.04
Indian wolf (N = 2)	0.22
Grey wolf Xinjiang (China) (N = 2)	0.06
Iberian wolf (N = 1)	0.20

## 4.5 | Taxonomy and conservation

Our study adds to growing evidence that the Himalayan wolf merits taxonomic recognition (Aggarwal et al., 2007; Álvares et al., 2019; Sharma et al., 2004; Werhahn et al., 2018; Werhahn, Senn, et al., 2017). The lineage is monophyletic, inhabits a geographically distinct region and exhibits diagnostic genetic characteristics (i.e. high-altitude adaptation) not found in populations of their closest relatives. Álvares et al. (2019) recently recommended specific recognition for the African wolf (*Canis lupaster*; Viranta et al., 2017), which is as distinct genetically as the Himalayan wolf (Table 3). In addition, consistent morphological (Hodgson, 1847; Janssens, Miller, & Van Dongen, 2016) and vocal differences (Hennelly et al., 2017) have been noted between Himalayan and grey wolves (although a systematic morphological study is recommended). On the grounds of this diverse evidence, species-level recognition is supportable in terms of the Unified Species Concept (Queiroz, 2007), the Differential Fitness Species Concept (DFSC) (Hausdorf, 2011) and the Biological Species Concept (BSC; Mayr, 1942).

Regardless of its formal taxonomic status, the Himalayan wolf population fulfils the criteria to be designated an 'evolutionary significant unit' (ESU) based on its discrete distribution and genetic divergence according to several genetic markers (Conner & Hartl, 2004; Moritz, 1994). Appropriate scientific recognition, such as a new taxonomic classification and assignment on the IUCN Red List, could catalyse much needed research regarding population size, ecology and behaviour, and could serve to focus the attention of regional and international conservation bodies on the plight of the Himalayan wolves, their habitat and the imperilled ecosystem in which they live.

## ACKNOWLEDGMENTS

We thank Xinning Shao for providing Sichuan samples. We thank Xueyang Li, Jiahao Niu, Xiaoyu Li and Qi Lu for help of lab work. We thank staffs from Shanshui Conservation Center and Center for Nature and Society, PKU, including Peiyun Li, Jiagongzhala, Dawajiangcai, Genggaiyan, Meisuonancuo, Qiunidantu, Juan Li, Lan Wu, Mingyu Liu, Xiaoyu Li, Xueyang Li, Xiang Zhao and community monitors in Zadoi County, for samples collection in Sanjiangyuan. We thank Andrew Kitchener, Benjamin N. Sacks and Olaf R.P. Bininda-Emonds for advice on the nomenclature. We thank Wang Jun, Bai Defeng, Chen Pengju and Pan Guoliang of Beijing Forestry University, Wildlife Institute, for samples collection in TAR, Gansu, Qinghai and Xinjiang. We thank Rahim Kulenbekov and the UW Hall Conservation Genetics Research Fund for collection of samples and support of preliminary lab work for Kyrgyzstan and Tajikistan. We also thank Zhang Cheng Cheng, Aliana Norris, Sydney Greenfield, Bayaraa Muntsog, Peng Xiaoxu and Ma Bing of Beijing Forestry University, for their help with the amplification of hypoxia-related genes. GW was supported by an Oxford-Lady Margaret Hall-NaturalMotion Graduate Scholarship.

## DATA AVAILABILITY STATEMENT

The data for this study, including accession numbers for genetic sequences deposited on NCBI GenBank, are recorded in the Supporting Information.

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#### BIOSKETCHES

G. W. is a wildlife conservation researcher with a focus on carnivores in the Asian high altitudes. The research team comprises experts dedicated to carnivore research and conservation from across the study range. G.W. and H.S. conceived the ideas; G.W., Y.L., C.C., X.S., Q.L., N.K., S.K., T.R., Z.K. and K.K. collected the data; G.W., Y.L., L.A., Z.D., X.S., Q.L., J.J., A.M.S., D.K., H.K.C., M.G., J.K. and H.S. analysed the data; G.W. led the writing; Y.M., S.K., K.S., B.W., D.W.M., C.S. and H.S. supported the writing.

#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Werhahn G, Liu Y, Meng Y, et al. Himalayan wolf distribution and admixture based on multiple genetic markers. *J Biogeogr.* 2020;00:1–14. <https://doi.org/10.1111/jbi.13824>